

Ectopic expression of class 1 *KNOX* genes induce adventitious shoot regeneration and alter growth and development of tobacco (*Nicotiana tabacum* L) and European plum (*Prunus domestica* L)

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Abstract Transgenic plants of tobacco (*Nicotiana tabacum* L) and European plum (*Prunus domestica* L) were produced by transforming with the apple class 1 *KNOX* genes (*MdKN1* and *MdKN2*) or corn *KNOX1* gene. Transgenic tobacco plants were regenerated in vitro from transformed leaf discs cultured in a medium lacking cytokinin. Ectopic expression of *KNOX* genes retarded shoot growth by suppressing elongation of internodes in transgenic tobacco plants. Expression of each of the three *KNOX1* genes induced malformation and extensive lobing in tobacco leaves. In situ regeneration of adventitious shoots was observed from leaves and roots of transgenic tobacco plants expressing each of the three *KNOX* genes. In vitro culture of leaf explants and internode sections excised from in vitro grown *MdKN1* expressing tobacco shoots regenerated adventitious shoots on MS (Murashige and Skoog 1962) basal medium in the absence of exogenous cytokinin. Transgenic plum plants that expressed the *MdKN2* or corn *KNOX1* gene grew normally but *MdKN1* caused a significant reduction in plant height, leaf shape and size and produced malformed curly leaves. A high frequency of adventitious shoot regeneration (96%) was observed in cultures of leaf explants excised from corn *KNOX1*-expressing transgenic plum shoots. In contrast to *KNOX1*-expressing tobacco, leaf and internode explants of corn *KNOX1*-expressing plum required synthetic cytokinin (thidiazuron) in the culture medium to induce adventitious

shoot regeneration. The induction of high-frequency regeneration of adventitious shoots in vitro from leaves and stem internodal sections of plum through the ectopic expression of a *KNOX1* gene is the first such report for a woody perennial fruit trees.

Keywords Adventitious shoot regeneration · Tobacco · Plum · Corn and apple *KNOX1* genes · Altered leaf morphology

Introduction

Adventitious shoot regeneration from in vitro-cultured explants is an important requirement for producing transgenic plants of most crop species. While most herbaceous species regenerate adventitious shoots from cultured explants when exposed to synthetic plant growth regulators, explants from perennial fruit trees are generally recalcitrant to regenerate adventitious shoots (Scorza 2001; Srinivasan et al. 2005). In these recalcitrant species a different strategy, one that utilizes the ectopic expression of genes involved in meristem and shoot apical meristem (SAM) formation, and development may provide a more successful approach. Work with *Arabidopsis thaliana* has revealed that shoot apical meristem formation and maintenance are regulated by a number of key genes, including the meristem identity genes, such as the class I *KNOX* (*KNOTTED-1-like HOMEOBOX*) genes, which belong to a homeobox gene family consisting of *STM*, *KNAT1*, *KNAT2* and *KNAT6* (Hake et al. 2004), the ortholog *KNOX1* gene in corn (Vollbrecht et al. 1991), and *OSH1* gene from rice (Matsuoka et al. 1993).

The *KNOX* genes are versatile regulators of plant development and diversity (Hake et al. 2004; Hay and

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Tsiantis 2010). Class 1 *KNOX* genes have been shown to control an indeterminate state of plant cells within SAMs (Doerner 2003). Mis-expression or over-expression of *KNOX1* genes results in adventitious meristem formation and alters plant morphology by switching cell fate from determinate into indeterminate (Sinha et al. 1993). Bharathan et al. (2002) showed that *KNOX* proteins are associated with complex leaf primordia and that their absence is associated with simple leaf primordia. Besides maintaining SAM function intact, *KNOX1* protein is also involved in producing variety of leaf shapes (Uchida et al. 2010). *KNOX* proteins activate cytokinin biosynthesis and decrease gibberellin accumulation in plants (Hewelt et al. 2000; Rosin et al. 2003; Hay et al. 2004; Yanai et al. 2005; Bolduc and Hake 2009). Adventitious shoot regeneration from in vitro cultured explants is a result of conversion of cells in determinate organs such as cells in leaves or stems into indeterminate cells. This conversion process is promoted by overexpression of both *KNOX1* genes as well as by cytokinin overproduction or supplementation. Adventitious shoot meristem formation has been induced by ectopic overexpression of corn and other *KNOX1* genes both in *Arabidopsis* and tobacco (Chuck et al. 1996; Sinha et al. 1993; Hake et al. 2004). Since upregulation of cytokinin biosynthesis and downregulation of active gibberellin are favorable conditions for adventitious shoot regeneration from in vitro-cultured explants, we used *KNOX1* genes to induce adventitious shoot regeneration in tobacco and plum.

In this report, we describe the effects of ectopic expression of corn *KNOX1* and apple *KNOX1* (*MdKN1* and *MdKN2*) genes on the growth and development of transgenic tobacco and plum. We show that leaves excised from these transgenic plants are able to produce adventitious shoots in vitro in the absence of exogenous cytokinin (tobacco) which is normally necessary for shoot regeneration, or with exposure to cytokinin that would normally produce no adventitious shoots (plum).

Materials and methods

Cloning of apple *KNOX1* genes

Total RNA was isolated from young leaves of 4–6 weeks old in vitro grown apple rootstock cultivar M26 using the RNeasy Plant Mini Kit (Qiagen). Genomic DNA was removed from the RNA sample with DNase treatment using the DNA-free kit (Ambion, Austin, TX, USA). About 5 µg of the treated RNA was converted into cDNA using the Superscript III cDNA synthesis kit (Invitrogen, San Diego, CA, USA). About 100 ng of the cDNA was used as templates for amplification of approximately 1.5 kb *MdKN1* and *MdKN2* gene fragments with the primer pair

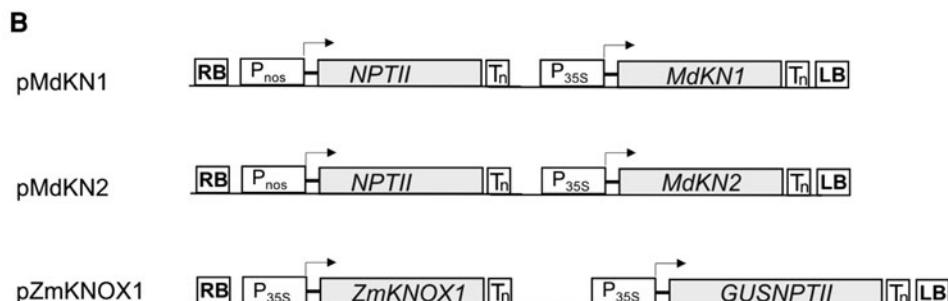
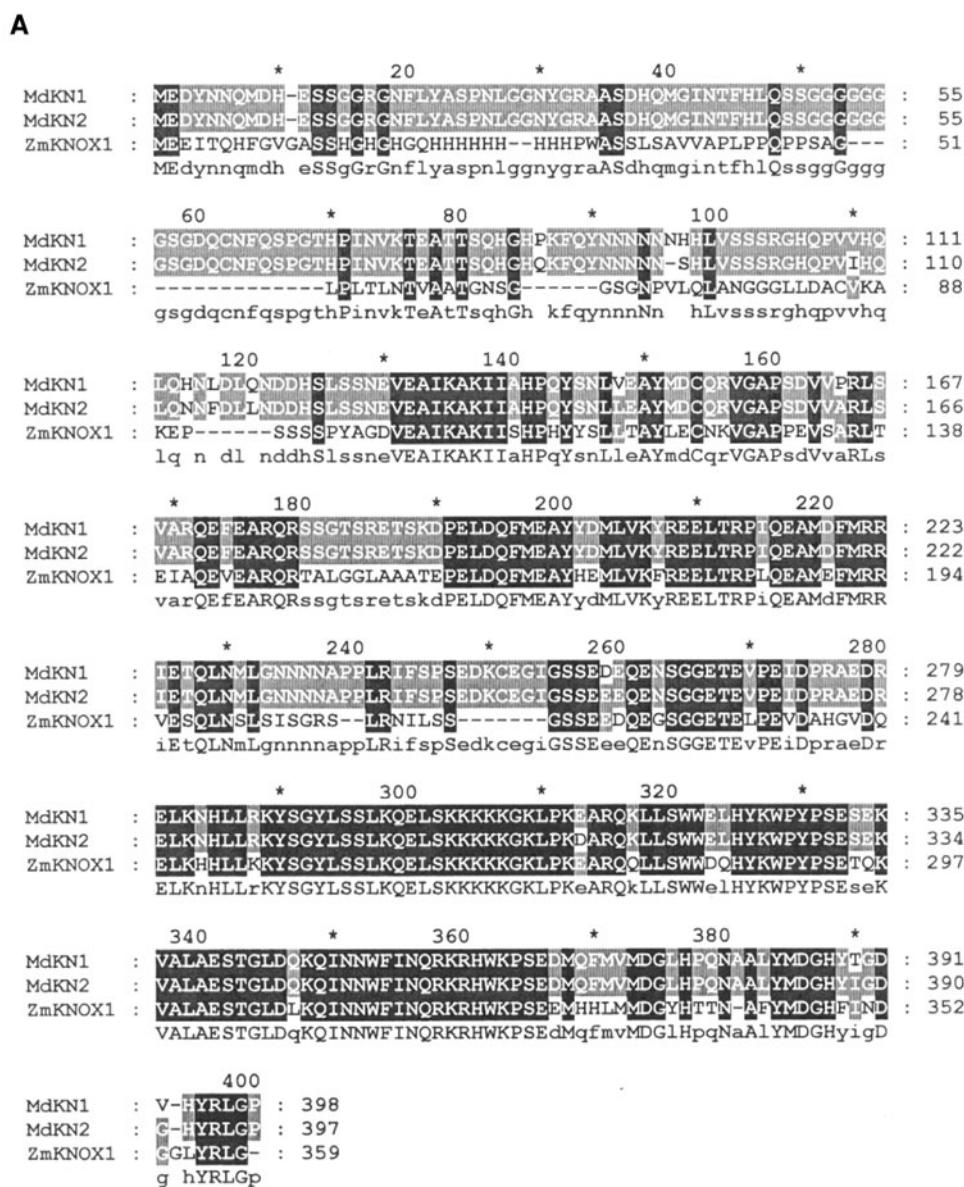
MdKN2U128 (CAT GCT TAA TTC CTT GTT TGG ATC CAC TTC CT) and *MdKN12U650* (GTCATCTGTTCT TCATCAAGAG). The same primer pair amplified both *MdKN1* and *MdKN2* cDNA gene fragment because the regions that the primer pair amplifies are highly conserved between *MdKN1* and *MdKN2* (Watillon et al. 1997). PCR amplification conditions include initial denaturing of DNA at 95°C for 2 min, followed by 35 cycles of amplification with 1 min at 95°C, 1 min at 60°C and 2 min at 72°C. The amplified gene fragments were confirmed by sequencing and they were inserted between a *CaMV35S* promoter and *Nos* terminator to create binary constructs pR2564 (*MdKN1*) and pR2563 (*MdKN2*). The corn *KNOX1* gene construct (35S::corn *KNOX1*) was kindly provided by Yi Li (Department of Plant Science, University of Connecticut, Storrs, CT 06269). Full length cDNA of all three *KNOX1* genes were cloned into pBIN19 binary vector (Bevan 1984) and used for this study. The alignment of deduced amino acid residues of *MdKN1*, *MdKN2* and corn *KNOX1* (*ZmKN1*) showed that *MdKN1* and *MdKN2* proteins share 96% identity, while the corn *KNOX1* shares only 49–50% identity with these two apple *KNOX1* proteins (Fig. 1a). The 35S::*MdKN1*, 35S::*MdKN2* and 35S::corn *KNOX1* constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101 (Holsters et al. 1980) for plant transformation. The apple *KNOX1* gene constructs contain 35S::*NPTII* for kanamycin selection, while the corn *KNOX1* construct contains 35S::*GUS-NPTII* fusion gene for selection (Fig. 1b).

Tobacco transformation

Leaf discs (5 mm diameter) excised from in vitro-germinated seedlings of *N. tabacum* cv. ‘Wisconsin 38’ were transformed with individual *KNOX1* genes. Transgenic shoots were selected on MS (Murashige and Skoog 1962) basal medium or MS medium containing 5 µM benzyladenine (BA) and 200 mg/l kanamycin and 500 mg/l cefotaxime, and rooted in vitro. The transgenic plants were acclimated in a growth chamber (20°C; 70 µmol photons m² s⁻¹ light intensity, 16 h light/8 h dark photoperiod; 70% relative humidity) and planted in 6 inch pots containing Metro-Mix 510 soil mix (Sun Grow Horticulture Inc., Washington State, USA) and were grown in the temperature-controlled greenhouse under natural lighting. Data were collected on the growth and development of control and *KNOX1* gene expressing tobacco plants, including plant height, morphology of leaves and time of flowering. Seeds were collected from self-pollinated transgenic plants for germination assays. To determine the presence of *KNOX1* transgene in the tobacco, polymerase chain reaction (PCR) analyses were performed using vector-specific primers with DNA extracted from the leaves sampled from both in vitro multiplied and greenhouse grown transgenic and control tobacco plants.

Fig. 1 Analyses of apple and corn *KNOX1* homologues.

a Multiple alignment of amino acid sequences of two apple class 1 *KNOX* paralogues, MdKN1 (Z71878) and MdKN2 (Z71979), with ZmKNOX1 (cornKNOX1-X61308). Numbers in parenthesis are GenBank accession numbers. The conserved amino acid residues among them are shaded with black or gray. All function- or amino acid- identical residues among three *KNOX1* homologues are shaded in black while two identical residues in gray. **b** Schematic diagrams of gene constructs. All three *KNOX1* genes that were driven by *CaMV35S* promoter were inserted into pBIN19 vector with *NPTII* or *GUS-NPTII* fusion selection marker genes. The arrow above each gene fusion denotes transcription initiation sites and direction. RB right border of T-DNA, LB left border of T-DNA, *P_{35S}* 35S promoter of cauliflower mosaic virus, *P_{nos}* promoter of nopaline synthesis gene, *T_n* transcription terminus of nopaline synthesis gene

**Plum transformation**

To transform plum, zygotic embryos were extracted from surface-sterilized seeds of plum cv. ‘BlueByrd’. Hypocotyl sections excised from the embryos were transformed with *Agrobacterium tumefaciens* strain GV3101 containing

35S::MdKN1, 35S::MdKN2 or 35S::corn *KNOX1* gene. The transformed hypocotyl sections were cultured in vitro and the transgenic shoots were regenerated, in the antibiotic selection medium containing 3/4 MS salts and vitamins, 7.5 µM thidiazuron (TDZ), 20 g/l sucrose, 7 g/l bacto agar (Becton–Dickinson and company, Sparks,

Maryland, USA), 80 mg/l kanamycin and 300 mg/l timentin (antibiotics). Transgenic plum shoots were multiplied in medium containing 3/4 MS salts and vitamins, 3 µM BA, 20 g/l sucrose, antibiotics and 7 g/l bacto agar. The plantlets were rooted in medium containing 1/2 MS salts and vitamins, 20 g/l sucrose, 5 µM α -naphthalene acetic acid, 0.1 µM kinetin, 40 mg/l kanamycin, and 7 g/l bacto agar and acclimated in the growth chamber. Rooted plantlets were planted in soil mix in 6 inch pots, and were grown in the greenhouse as described by Petri et al. (2008). Data on the height of control and transgenic plum plants, leaf length and leaf width were collected after 1 year of growth in the greenhouse. To determine the presence of corn and apple *KNOX1* genes in the transgenic plums, PCR analyses were performed using the vector-specific primers of *KNOX1* genes and *NPT II* gene with DNA extracted from the actively growing leaves of transgenic and control plum shoots multiplied both in vitro as well as from the leaves of greenhouse grown plum plants.

Adventitious shoot regeneration in vitro from *KNOX1* gene expressing tobacco and plum leaves

Leaf discs and stem sections excised from transgenic tobacco plants multiplied in vitro were cultured on MS basal medium to study the efficiency of adventitious shoot regeneration in the absence of cytokinin. To study the regenerative competence of *KNOX1* transgenic plum, recently unfolded young leaves from the first node, as well as the leaves excised from the second, third and the fourth nodal positions from the shoot tip of control and *KNOX1* gene expressing plants, were exposed to adventitious shoot regeneration-inducing media. Eighty leaf explants were excised from each leaf position. After making three scalpel-wounds across the midrib, the leaf explants were cultured in Petri plates, first in callus induction medium containing QL (Quoirin and Lepoivre 1977) macronutrients, DKW (Driver and Kuniyuki 1984) micronutrients and vitamins, 9 µM TDZ, 9 µM 2,4-dichlorophenoxy acetic acid, 30 g/l sucrose and 7 g/l Bacto agar and incubated for 4 days in dark. The leaf explants were then transferred to adventitious shoot induction medium in Petri plates containing QL macronutrients, DKW micronutrients and vitamins, 9 µM TDZ, and 60 µM silver thiosulfate, 30 g/l sucrose and 7 g/l Bacto agar and incubated in dark for 10 days (Petri and Scorza 2010). Adventitious shoot regeneration was first observed within a week when the leaf explants were exposed to light (16 h light/8 h dark period, 45–55 µ Einstein m⁻² s⁻¹ light intensity) and incubated at 24°C. Data on adventitious shoot regeneration were collected 1 month later. Explants of internode and root cross sections were also cultured as described above to evaluate their competence to form adventitious shoots. All

leaf, internode and root explants were cultured in media with or without the addition of the synthetic cytokinin, TDZ. Transgenic and control plum shoots were rooted in vitro, acclimated in the growth chamber, and planted in soil mix in 6 in pots and were grown in a temperature-controlled greenhouse under natural light as described above to evaluate their growth and development. All data were subjected to analysis of variance (ANOVA) and Bonferroni least significant differences (LSD) of means at $P = 0.05$ using the SAS statistical program (SAS Institute Inc. 2008).

Results

PCR analyses with vector-specific primers of *KNOX1* genes and *NPTII* genes using the DNA extracted from in vitro grown shoots and greenhouse grown transgenic tobacco and plum plants showed the presence of corn or apple *KNOX1* genes (data not shown).

Transformation of *Nicotiana tabacum* with *KNOX1* genes

Ectopic expression of both corn and apple *KNOX1* genes induced adventitious shoot regeneration from leaf discs of tobacco when cultured on MS basal medium (MSBM) indicating that *KNOX1* genes were active in inducing cytokinin biosynthesis as soon as tobacco leaf disc were transformed which promoted adventitious shoot initiation, but the frequency of transformation and the number of transgenic shoots per leaf disc was significantly lower when compared with shoot regeneration from leaf discs cultured on MSBM with cytokinin (5 µM BA). Untransformed control leaf discs atrophied without regenerating any adventitious shoots when exposed to antibiotic selection (200 mg/l kanamycin) medium even in the presence of 5 µM BA (Table 1).

Regeneration from *N. tabacum* ectopically expressing *KNOX1* genes

Adventitious shoots regenerated in vitro from whole leaves that were excised from 35S::*MdKN1*, 35S::*MdKN2* or 35S::corn *KNOX1* transgenic shoots and cultured on MSBM (Fig. 2a). These shoots produced extensive root systems on the same medium. Both abaxial and adaxial sides of leaves initiated adventitious shoots. Similarly, stem sections of transgenic *KNOX1* tobacco plants produced four adventitious shoots from each stem section after making four round bumps on the stem (Fig. 2b). In addition to regeneration from excised leaves and stem sections, in situ regeneration of shoots was observed from intact leaves and roots of transgenic plantlets grown in vitro (Fig. 2c, d).

Table 1 Adventitious shoot regeneration from leaf discs of ‘Wisconsin’ tobacco transformed with corn *KNOX1*, *MdKN1* and *MdKN2* genes and cultured on MS basal medium (MSBM) or MSBM + 5 µM BA supplemented with 200 mg/l kanamycin and 500 mg/l cefotaxime

Gene construct	Medium	Percent leaf discs regenerated	Mean number of adventitious shoots/leaf disc ± SE
Control	MSBM	0	0
Corn <i>KNOX1</i>	MSBM	44	5.08 ± 1.28a
<i>MdKN1</i>	MSBM	36	4.68 ± 1.33a
<i>MdKN2</i>	MSBM	28	4.08 ± 1.37a
Control	MSBM + 5BA	0	0
Corn <i>KNOX1</i>	MSBM + 5BA	90	14.44 ± 1.00a
<i>MdKN1</i>	MSBM + 5BA	90	11.08 ± 1.02b
<i>MdKN2</i>	MSBM + 5BA	75	8.64 ± 1.00b

Data were collected 2 months after transformation. Each value is a mean of 25 leaf discs ± standard error (SE) of mean

* Any two means with no letter in common are significantly ($P = 0.05$) different by Bonferroni LSD method

These observations indicate that constitutive over-expression of all three *KNOX1* genes made all organs of the tobacco competent to regenerate adventitious shoots both *in situ* as well as after excision and culture as explants.

Overexpression of *KNOX1* genes drastically altered the growth and development of tobacco plants (Table 2). All *KNOX1* plants were significantly shorter than controls due to suppression of internode elongation, however, the number of nodes and leaves produced were similar to that produced by control plants (Table 2; Fig. 2e–g). Alteration of leaf morphology was the most noticeable effect of ectopic expression of all three *KNOX1* genes. However, the pattern of leaf lamina modification varied between the *KNOX1* genes. Control tobacco leaves are relatively large and oval in shape (Fig. 2e) but leaves of *KNOX1* over-expressing transgenic tobacco were small and malformed (Fig. 2f, g). As compared with *MdKN1* plants which produced distorted laminae, plants expressing *MdKN2* showed severe alteration of plant growth and produced lobbed laminae and small leaves. Corn *KNOX1* caused little alteration in plant growth and lamina expansion as compared with *MdKN1* and *MdKN2* expressing plants which resulted in significant reduction in leaf size (Table 2). All *KNOX1* plants flowered 6–11 days earlier than controls. Fruit set and seed development in all *KNOX1* plants were normal. Percent germination of seeds from *KNOX1*-expressing tobacco plants was normal (data not shown).

Regeneration from *Prunus domestica* ectopically expressing *KNOX1* gene

Plum plants displayed altered leaf morphology when transformed with *MdKN1*, while the plants expressing corn

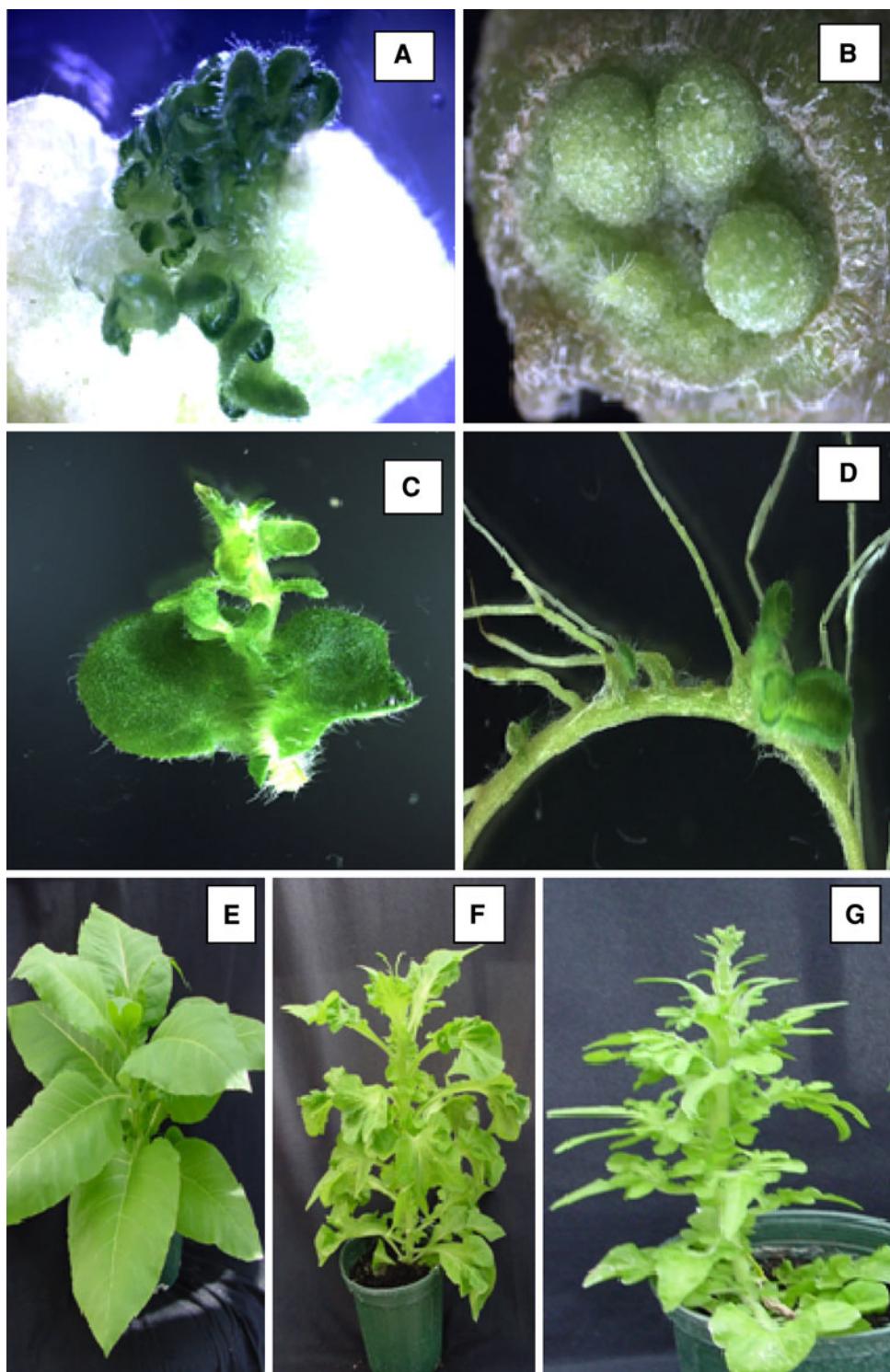
KNOX1 and *MdKN2* genes showed no change in leaf morphology (Fig. 3). Growth and development of corn *KNOX1* and *MdKN2*-expressing plants in the greenhouse was similar to control plants, but most of the *MdKN1* plum plants were dwarf and produced round or distorted and diminutive dark green curly leaves as compared with control leaves, which were lighter green and lanceolate in shape (Fig. 3b, c). Height of plants, number of leaves, and length and width of leaves are all significantly less in *MdKN1* plum plants as compared with the *MdKN2* and corn *KNOX1* plum plants (Table 2; Fig. 3c). Three classes of *MdKN1* plum plants were observed: dwarf (<12 cm), semi dwarf (13–46 cm) and tall (>120 cm) (Table 2; Fig. 3b).

Leaf explants sampled from all four nodal positions of corn *KNOX1*-expressing plum shoots regenerated adventitious shoots when cultured on plum regeneration medium containing 9 µM TDZ. There was no significant difference in the mean number of adventitious shoot regeneration between the nodal positions of leaf explants (Fig. 3d; Fig. 4). Up to 96% of the leaf explants of corn *KNOX1*-expressing plum shoots regenerated adventitious shoots, while only 56% of the curly leaf explants from *MdKN1*-expressing shoots produced adventitious shoots (9.26 ± 1.43) in the same medium. Up to 38 adventitious shoots were produced *in vitro* from single leaf explant excised from corn *KNOX1* gene expressing shoots. No adventitious shoot regeneration was observed in cultures of leaf explants excised either from *MdKN2* gene expressing plants or from untransformed control plum shoots. Most of the adventitious shoots were regenerated from the callus produced by leaf midribs and petioles. About 20% of the internode sections excised from corn *KNOX1*-expressing shoots regenerated adventitious shoots (Fig. 3e), while no shoots were regenerated from root explants from corn *KNOX1* plum plants. There was no regeneration from leaf explants from corn *KNOX1* plum shoots in the absence of TDZ in the medium. All adventitious shoots rooted readily *in vitro* (Fig. 3f) and they were acclimatized in a growth chamber and planted in the greenhouse (Fig. 3g).

Discussion

Constitutive over-expression of *KNOX1* genes in tobacco and plum generally resulted in distorted leaves and caused ectopic adventitious shoot formation with some important differences related to the specific plant species and *KNOX1* source. This general finding is in agreement with the observed expression of most class 1 *KNOX1*-like genes in other species (Hake et al. 2004; Hay and Tsiantis 2010). The natural endogenous expression of *KNOX1* genes in plants is restricted to SAM, and their expression is

Fig. 2 Adventitious shoot regeneration in situ and from in vitro cultured leaf explants, stem sections and roots of *KNOX1* gene expressing tobacco plantlets cultured on MSBM and growth and development of transgenic tobacco plants in greenhouse. **a** De novo adventitious shoot formation from a leaf explant. **b** Shoot primordial structures from an internode section. **c** In situ adventitious shoot formation from a leaf. **d** In situ adventitious shoot initiation from intact roots. **e** Control tobacco plant. **f** Ectopic expression of *MdKNI* caused short plant with crinkled leaves. **g** *MdKN2*-expressing plant showing dissected compound leaves



excluded from incipient leaf primordia (Doerner 2003). In simple-leaved species such as tobacco and plum, native *KNOX1* genes are expressed in the SAM and unexpanded axes and are downregulated after leaf initiation (Bharathan et al. 2002). Constitutive expression of *KNOX1* genes under the control of the 35S CaMV promoter induced SAM development and in most cases, depending on the plant

species and *KNOX1* gene source, altered the pattern of leaf development and induced leaf lobing. Natural leaf dissection may be the result of reactivation of meristematic genes within developing leaf primordia of certain species. This idea is based on the observation that in simple leaf species such as maize, *Arabidopsis*, snapdragon and tobacco, class I *KNOX* genes are expressed in the SAM and

Table 2 Growth and development of control and transgenic tobacco and plum plants ectopically expressing corn *KNOX1*, *MdKN1* or *MdKN2* genes

<i>KNOX1</i> gene	Mean height of plant	Mean number of leaves/plant	Leaf length	Leaf width
Tobacco				
Control	84.6 ± 1.56a	23.7 ± 0.58a	32.6 ± 0.89a	16.7 ± 0.32a
Corn <i>KNOX1</i>	63.1 ± 2.10b	22.9 ± 0.60a	20.9 ± 0.64b	10.2 ± 0.61b
<i>MdKN1</i>	54.3 ± 1.50c	22.3 ± 0.56a	9.2 ± 0.73c	6.1 ± 0.39c
<i>MdKN2</i>	48.8 ± 0.95d	23.1 ± 0.58a	8.8 ± 1.25c	3.4 ± 0.60d
Plum				
Control	121.5 ± 4.10a	73.4 ± 1.77a	7.4 ± 0.16a	6.7 ± 0.16a
Corn <i>KNOX1</i>	104.3 ± 2.96b	67.5 ± 3.12ab	7.3 ± 0.27a	5.9 ± 0.22b
<i>MdKN1</i>	59.3 ± 6.67c	36.3 ± 2.94c	4.9 ± 0.51b	3.2 ± 0.27c
<i>MdKN2</i>	97.2 ± 5.00b	63.4 ± 3.44b	7.8 ± 0.22a	5.3 ± 0.28b

The tobacco plants were grown until flowering, while the plums were grown for a year in the greenhouse. Height of plants, length and width of leaves are in centimeters. Each value represents mean of ten plants ± standard error (SE) of mean

* Any two means with no letter in common are significantly ($P = 0.05$) different by Bonferroni LSD method

Fig. 3 Growth and development of 1-year-old transgenic plum plants ectopically expressing corn and apple *KNOX1* genes and adventitious shoot regeneration from leaf explants of corn *KNOX1*-expressing plum shoots. **a** Control plum plant. **b** Modification of growth and development of plum plants due to overexpression of *MdKN1*. **c** Large control leaf (left) and reduction in leaf size and distortion of leaf shape due to overexpression of *MdKN1*. **d** High frequency adventitious shoot regeneration in the cultures of leaf explants excised from nodes 1 to 4 of corn *KNOX1*-expressing shoots. Leaf position 1 is the youngest unfurled leaf near the shoot tip and the leaf positions 2, 3 and 4 are older leaves excised from the nodes immediately below the leaf position one. **e** Adventitious shoot regeneration in an internode explant. **f** In vitro-rooted adventitious shoots. **g** Acclimation of rooted plum plantlets in the greenhouse



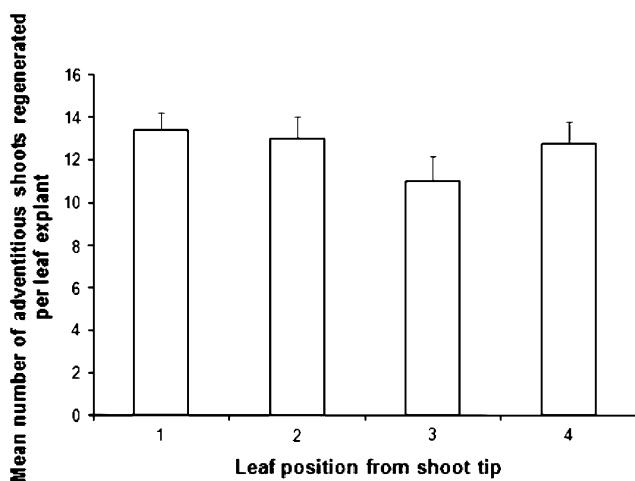


Fig. 4 Histogram showing the mean \pm standard error of adventitious shoot regeneration in vitro from plum leaf explants excised from 1 to 4 leaf positions in corn *KNOX1*-expressing shoot cultures. Leaf positions are same as described in Fig. 3. Eighty leaf explants were excised from each leaf position and used for adventitious shoot regeneration

excluded from leaves (Hay and Tsiantis 2010), whereas many dissected leaf angiosperms such as tomato and palm express *KNOX* genes in leaves and *KNOX* activity in leaves is required for leaflet formation (Chuck et al. 1996; Hay et al. 2004; Müller et al. 2006; Jouannic et al. 2007; Uchida et al. 2010). Additionally, overexpression of *KNOX* genes in tomato results in the production of super-dissected leaves (Hareven et al. 1996; Janssen et al. 1998; Shani et al. 2009, 2010), indicating that leaf dissection is sensitive to the levels of *KNOX* expression. However, the pattern and degree of distortion caused by ectopic expression of different *KNOX1* genes varied in tobacco.

In tobacco, corn *KNOX1* gene expression reduced lamina size, but there was a little distortion in lamina development. *MdKN2* produced small leaves and extensive lobe development. In the case of *MdKN1*, leaves were twisted and had a round shape as compared to a flat, elliptical shape of control leaves. Transgenic plum plants expressing corn *KNOX1* or *MdKN2* genes did not display reduced shoot growth or leaf distortion, but *MdKN1*-expressing plants were dwarf with twisted small, curly and rounded leaves (Fig. 3b, c). Watillon et al. (1997) suggested that *MdKN1* and *MdKN2* are two distinct copies of the same gene. Alignment of aminoacids of corn *KNOX1* (*ZmKNOX1*) with apple *MdKN1* and *MdKN2* proteins also showed 96% conservation between the apple *KNOX1* proteins, but there was only 49–50% conservation between corn *KNOX1* and apple *KNOX1* proteins (Fig. 1a). Differential ectopic expression of apple and corn *KNOX1* genes in plum might be due to the differences in polypeptide sequences between these *KNOX1* proteins. Recently, it has been reported that class 1 *KNOX* gene

(*KNOPE1*) cloned from another close-related *Prunus* fruit species, peach (*Prunus persica*) also induced lobed leaves when ectopically expressed in *Arabidopsis* (Testone et al. 2008). The involvement of ectopically expressing *KNOX1* gene in plum in the switch between differentiated and undifferentiated cell fate suggests that maintenance of reservoirs of meristematic cells is under the control of *KNOX1* class of genes both in herbaceous and woody perennial plants, as observed previously in apple (Watillon et al. 1997) and poplar (Groover et al. 2006).

It has been widely reported that *KNOX1* genes upregulate cytokinin biosynthesis and downregulate gibberellin biosynthesis (Kusaba et al. 1998; Rupp et al. 1999; Hewelt et al. 2000; Frugis et al. 2001; Rosin et al. 2003; Hake et al. 2004; Jasinski et al. 2005; Sakamoto et al. 2006; Shani et al. 2006, 2010; Hay and Tsiantis 2010). In *Arabidopsis* the *KNOX1* gene shoot meristemless (STM) protein induces expression of the cytokinin biosynthetic pathway gene isopentenyl isomerase (*AtIPI*) within 2 h after induction (Jasinski et al. 2005; Yanai et al. 2005). Ectopic expression of corn *KNOX1* gene made tobacco plants cytokinin-autotrophic (Hewelt et al. 2000). Adventitious shoot regeneration in vitro from leaf discs, whole leaf, stem sections and roots of transgenic tobacco constitutively expressing corn or apple *KNOX1* genes on MS basal medium (Table 1; Fig. 2a–d) is clear indication of cytokinin-autotrophy. Leaf curling observed in the ectopically overexpressing *MdKN1* plum (Fig. 3b, c) is similar to the symptoms produced by the leaf curl disease (*Taphrina deformans*) in the related fruit species, peach (*Prunus persica*). Testone et al. (2008) showed that these curly-leaf disease symptoms could be mimicked in peach by the overexpression of the peach *KNOPE1* gene which induced de novo zeatin synthesis. In addition to upregulation of cytokinin biosynthesis, suppression of GA 20-oxidase gene expression by the *KNOX* protein has been reported in both dicot and monocot plants (see reviews by Hake et al. 2004; Hay and Tsiantis 2010). Bolduc and Hake (2009) showed that the corn *KNOX1* protein negatively modulates GA accumulation through direct regulation of the GA catabolism gene *ga2ox1*. These results indicate that plant meristems need high cytokinin and low gibberellin levels to maintain their growth and development activity and that *KNOX* proteins act as central regulators to control these phytohormones at adequate balance, regardless of the differences in organization between monocots and dicots (Sakamoto et al. 2006; Hay et al. 2002; Chen et al. 2004; Hake et al. 2004). Based on these overwhelming earlier findings, we suggest that reduction of shoot elongation growth, modification of leaves and adventitious shoot regeneration in tobacco and plum reported here might have been the result of simultaneous repression of GA biosynthesis and elevation of de

novo cytokinin biosynthesis due to constitutive ectopic expression of *KNOX1* genes.

Although constitutive expression of the *KNOX1* gene in tobacco stimulated the regeneration of adventitious shoots in the absence of exogenous cytokinin, *KNOX1*-expressing plum leaf explants required the addition of synthetic cytokinin such as TDZ to induce adventitious shoot regeneration. Ectopic *KNOX1* gene expression induces overproduction of natural cytokinins (Yanai et al. 2005; Hay et al. 2004) which induces adventitious shoot regeneration in herbaceous species such as *Arabidopsis* or tobacco in hormone-free medium (Chuck et al. 1996; Sinha et al. 1993), but woody perennials such as plum require TDZ to induce adventitious shoot regeneration in addition to ectopic expression of the *KNOX1* gene (Figs. 3d, 4). Clearly, this gene presents new possibilities for the stimulation of adventitious shoot organogenesis and therefore the application of genetic engineering approaches to plum and other recalcitrant woody species. However, further studies are needed to elucidate the molecular and biochemical mechanism of induction of regenerative competence in the corn *KNOX1*-expressing transgenic plums.

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